MEMORANDUM

SUBJECT: Human Health Hazard Assessment for TERA R18-01 – **NOT CBI**

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SUMMARY

There is low concern for human health effects resulting from the use of these two intergeneric strains of *Chlorella sorokiniana,* PACE_ Cs1412_SNRK2 (containing a pyrroline-5-carboxylate synthase for normal populations in terms of pathogenicity, toxicity, and allergenicity. There is also low concern for pathogenicity to the susceptible subpopulation of immunocompromised individuals. There is low concern for toxicity to humans as *Chlorella sorokiniana* is not known to produce any phycotoxins. There is low concern for allergenicity to the general population. *Chlorella* are one of the most prevalent algae in the environment, occurring in fresh water, marine water, and edaphic environments. Thus, humans are respiring them routinely.

I. INTRODUCTION

USEPA has received a designated non-CBI TERA application similar to one received last year, case [redacted], for the same species *Chlorella sorokiniana* but for a different strain, this time *C. sorokiniana* PACE_Cs1412_SNRK2 (from now on known as Cs1412_SNRK2). The submitter identifies the parental organism as *Chlorella sorokiniana* DOE1412. This strain was isolated from the field by Juergen Polle in 2013 (UTEX website, accessed 09/2018) and deposited to the CUNY collection. Subsequently, the National Alliance for Advanced Biofuels and Bio-Products (NAABB) consortium, after a screening process has made 30 of their best performing strains, including DOE1412, made this strain available to the public through UTEX. These UTEX strains have been well characterized by DOE for lipid production and growth kinetics. UTEX and DOE, describe the strain as a high temperature freshwater strain (cold-sensitive) with a maximum growth temperature of 42 deg C. The strain is also referenced as DOE1412, NAABB 1412 and NAABB 2412.

Chlorella in general demonstrate rapid growth under phototrophic (Janssen et al., 1999) and mixotrophic (Wan et al., 2012) conditions. The genus also exhibits high heat tolerance with growth up to 42ºC, making it one of the most used microalga species for large scale industrial/bioremediation applications (Zeraatkar et al., 2016).

PACE_Cs1412_SNRK2 contains an introduced gene, sucrose non-fermenting (SNF) related kinase gene, *SNRK2*, which is part of the serine/threonine kinases (Kertesz et al., 2002) and plays a key role in sugar metabolism in plant and animal kingdoms and controls multiple growth and metabolic processes. The submitter is utilizing *SNRK2* gene to improve photosynthetic efficiency and biomass in the recipient organism The gene was synthesized in its native state (only the coding regions) without codon optimization and cloned into the PACE *Chlorella* plasmid vector. The regulatory elements used to express the SNRK gene are the *psaD* (a photosynthesis-related gene) and *actin* promoters and terminators, both of which are endogenous to the recipient microorganism.

II. GENETIC MODIFICATIONS

A. RECEPIENT MICROORGANISM

The recipient strain for this project will be *C. sorokiniana* DOE1412. This organism can be identified by running a whole cell approach to PCR with the specific primers developed for allowing discrimination from other *Chlorella sp*., even specific strains within species. The *C. sorokiniana* 1412 specific primers are a) FWD 5' GCGAAGAAGAAAATGTAAACTTATTAG 3' and b) Rev 5' CCATTCCAGTAATTGCTAAATCA 3'.

Of note and with respect to Figure 1 of the TERA application, Rosenberg et al. (2014) used strains of *C. variabilis* that cluster within the group that some suggest are the true *Chlorella.* The tree provided shows that *C. sorokiniana* clusters separately from *C. vulgaris* and *C. variabilis* strains, its closest neighbors in that study.

B. DONOR ORGANISMS

The intergeneric gene used to develop the strains in this TERA, the *SNRK2* gene was derived from a *Picochlorum soloecismus* strain, a genus of green algae in the class Trebouxiophyceae.

Picochlorum soloecismus is a halotolerant, fast-growing and moderate lipid producing microalga that has been evaluated as a renewable feedstock for biofuel production by the DOE (Gonzalez-Esquer et al., 2018).

The sucrose non-fermenting (SNF) related kinase gene, *SNRK2*, is part of the serine/threonine kinases (Kertesz et al., 2002) and plays a key role in sugar metabolism in plant and animal kingdoms and controls multiple growth and metabolic processes. The submitter is utilizing *SNRK2* gene to improve photosynthetic efficiency and biomass in the recipient organism The gene was synthesized in its native state (only the coding regions) without codon optimization and cloned into the PACE *Chlorella* plasmid vector. The regulatory elements used to express the SNRK gene are the *psaD* (a photosynthesis-related gene) and *actin* promoters and terminators, both of which are endogenous to the recipient microorganism.

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III. HUMAN HEALTH HAZARDS OF THE RECEPIENT MICROORGANISM

The genus *Chlorella* has been found throughout all of North America from tropical to arctic climates. *Chlorella* spp. are omnipresent in both aquatic and terrestrial environments (Hodac et al., 2016).

Like many other algae, *Chlorella* is an important primary producer and food source for higher trophic levels.

A. PATHOGENICITY

C. sorokiniana is capable of growth at human body temperature (37ºC) as it is one of the most heat tolerance species in the genus. However, there is no evidence in the literature that the species *C. sorokiniana* causes infections in humans. According to the submission, the American Type Culture Collection (ATCC) lists *C. sorokiniana,* along with 22 other strains of the genus *Chlorella,* as Biosafety Level (BL1) 1 organisms based upon the fact that these organisms are not known to cause disease in healthy humans.

Chlorellosis is the name of a rare *Chlorella* infection that has occurred in limited numbers in sheep and cattle, and in single cases in a human, dog, gazelle, beaver, camel, and fish (as summarized by Hart et al., 2014). Animals are infected by exposure of open wounds to contaminated water. In mammals this disease ranges from localized cutaneous infection, lymph node infection, or dissemination to multiple organs. However, in humans, the three reported cases were cutaneous infections (Jones et al., 1983; Yu et al., 2009; Hart et al., 2014). Chlorellosis in humans is extremely rare as there have been just three reported cases when the alga *Chlorella* is prevalent globally in fresh water lakes and rivers, in marine waters, and in soil.

The first case of chlorellosis in humans was described by Jones et al. (1983) where a 30-year-old woman developed persistent infection of a healing operative wound on her right foot after possible contamination by river water while canoeing. The wound was debrided two months later and the infection then treated with antibiotics and wound irrigation. The infection was persistent and healed completely only after 10 months.

The second case of *Chlorella* infection was an external infection found in the gangrene tissue from the right foot of a diabetic 59-year-old female (Yu et al., 2009). The *Chlorella* isolate was thought to be *C. saccharophila,* a *Chlorella* strain that uses glucose as a sole carbon source, grows at pH 2-3, and grows at temperatures up to 30ºC. The authors stated the strain "could not grow at 37°C in light or darkness. The results suggest that this strain may not normally invade tissues, but becomes established and grows on previously infected tissues of external body extremities where the temperature is somewhat lower than normal body temperature."

The most recent case of chlorellosis was reported in Australia in a 30-year-old man in a knee wound contaminated with fresh water dam water (Hart et al., 2014). He developed *Chlorella* and *Aeromonas hydrophila* infection within two days of exposure and the infection was aggressive and required debridement, negative pressure wound dressings, and antibiotics. The wound had healed by the third week with no further complications.

Altogether, the available evidence indicate a low concern for pathogenicity based on 3 identified instances of chlorella infection in humans.

B. TOXICITY

According to the submission, there are no reports in the literature that any *Chlorella* species, including *C. sorokiniana,* synthesizes or secrets phycotoxins. Recently, toxicity studies with *C. sorokiniana* revealed no toxicity either *in vitro* cytotoxicity assays or in subchronic toxicity studies in rats (Himuro et al., 2017).

Chlorella is a popular human nutritional supplement and extracts are used in skin care products. *Chlorella* sp. are generally regarded as safe (GRAS) for human consumption. *Chlorella* sp. and *C. protothecoides* flours have GRAS status (GRN 000330; GRN 000519). *Chlorella* has been proposed as a protein supplement for human consumption (Becker, 2007).
In humans, *Chlorella* sp. supplements have shown beneficial effects including improved immune responses, improved healing of the small intestine epithelium, antioxidant action and even anti-tumoral effects (Ramirez-Romero et al., 2010). *C. vulgaris* has been promoted as a prevention of anti-inflammatory responses (Hasegawa et al., 1999). Morin et al. (1980) have shown inhibitory effects of the unicellular alga *Chlorella* against murine sarcomas. *C. sorokiniana* was also found to activate dendritic cells resulting in T cells activation (Chou et al., 2012). Lastly, *C. sorokiniana* extract was shown to increase short term memory but may reduce social behavior in rats, with authors cautioning its use in natural supplements (Morgese et al., 2016).
Chlorella sp. has been used as a supplement to animal feed.

There is one study in the literature that reported cytotoxicity of algal dietary supplements consisting of a mixture of *Chlorella* sp. and the collective cell biomass from two cyanobacteria, *Arthrospira platensis* and *A. maxima* commonly referred to as *Spirulina* (Huessner et al., 2012). They found extracts from 13 commercially available products sold in Germany were cytotoxic in the A549 cell line with the *Spirulina* being more potent than *Chlorella*. This toxicity, however, was due to contamination of the cyanobacterial and algal cultures by microcystin, a potent toxin produced by the cyanobacterium *Microcystis.* The toxicity was not due to the microbes themselves. In addition, ingestion of algal dietary supplements is not relevant to human exposures in this small-scale field test.

Altogether the available evidence indicates a low concern for toxicity of Chlorella based on its use as a food supplement and skin care product.

C. ALLERGENICITY

Earlier literature suggested that airborne algae may be associated with respiratory problems (Woodcock 1948). Although the article stated that algal particles of 3-30 μm caused cough and nasopharyngeal burning in individuals frequenting the local beaches during onshore winds, it was later determined that the cause of the respiratory problems were brevitoxins, not the algae itself.

McGovern et al. (1965) were among the first to study the role of airborne algae in causing allergenicity. They stated that algae and cyanobateria usually constitute a minority of airborne bioaerosols compared to fungi, pollen, and bacteria. However, in certain cases the quantity of airborne algal particles can far exceed that of fungi spores and pollen grains (McGovern et al. (1965). Brown et al. (1965) found over 3000 algae/m3 in samples taken from a car moving through a dust cloud in Texas. They also said that the majority of airborne algae comes from soils. Schlichting (1969) calculated that since a human inhales about 7 L of air per minute, at least 2880 algal and cyanobacterial cells are inhaled per day. Thus, humans are routinely exposed to high numbers of algae on a daily basis.

Bernstein and Safferman (1966) tested two species of *Chlorella, C. vulgaris* and *C. pyrenoidosa,* two species of *Chlorococcum, C. botryoides* and *C. macrostigmatum*, *Scenedesmus basilensis,* and *Ankistrodesmus falcatus* var. *acicularis* for their allergenic potential in atopic patients, i.e., those with a genetic predisposition for developing allergic hypersensitivity reactions. Allergy can be

described as an immune response that is harmful or detrimental to the host. They found that of 79 atopic patients tested with algal extracts, 47 also gave positive skin reactions. However, non-atopic individuals did not show positive skin reactions. Additional tests with *C. vulgaris* for bronchial mucosa tests resulted in clinical wheezing. However, they tested only eight patients who had shown a pronounced skin reaction in earlier tests. Bernstein et al. (1969) tested the same six algal species for sensitization reactions in an animal model. However, the authors stated that neither their preliminary clinical studies (Bernstein and Safferman 1966) nor the current study established a definite causal role for green algae in human respiratory allergies.

Tiberg et al. (1995) tested children for allergy to *Chlorella* using the radioallergosorbent test (RAST) which is a blood test using a radioimmunoassay test to detect specific IgE antibodies to determine whether a subject is allergic to the substance. Skin prick tests (SPTs) were also conducted using a partially purified, lyophilized extract from the green alga *Chlorella homosphaera.* In addition, conjunctival provocation tests (CPT) were conducted with the *Chlorella* extract. No *Chlorella*-specific IgE antibodies were found in the sera from the 94 children from the general population (group 1 – no allergy symptoms). In a group of children that had been referred to an outpatient pediatric allergy clinic (group 2), nine of the 129 children had positive wheal reactions with the *Chlorella* extract in SPTs. Sera from seven of these children with positive SPTs results were available for analysis of IgE antibodies. Two of the seven were positive. Sera from four patients with negative SPTs showed negative IgE test using RAST. Seven of 23 mold-sensitive children (group 3) had positive SPTs to *Chlorella.* Six patients with SPT positive results and two of the 16 patients with negative SPT results had positive RAST results. All patients with positive SPT results showed some reaction in CPTs with *Chlorella* extract (5 mg dry weight/ml). The authors concluded that "The low degree of clinical sensitization to *Chlorella* found among nonatopic and atopic children in Sweden, the higher sensitization among atopic children with multiple sensitivities, the low levels of IgE antibodies against *Chlorella* as measured by RAST**,** and the great amount of protein material used to obtain a positive CPT result all indicate that *Chlorella* is a "weak" allergen" (Tiberg et al., 1995).

One database, the Allergome (Allergome), lists *Chlorella* as an allergen source. Based on a review of outdoor allergens Burge and Rogers (2000) stated that algae do not seem to be a source of major outdoor allergens. In a series of articles, ******

The submitters state that none of their workers have responded to *C. sorokiniana* adversely despite years of cultivation in both closed reactors and open ponds. Although they have only been growing *C. sorokiniana* outdoors for one year, they have been growing various strains of *Chlorella* for more than 10 years at the site.

Altogether the available data indicate a low concern for allergenicity based on the lack of positive data in the available databases for allergenic proteins and no available studies indicating that chlorella is a sensitizer.

D. OTHER EFFECTS

Chlorella has also been reported to cause photosensitization, which is development of abnormally heightened reactivity of skin or eyes to sunlight, in those who used *Chlorella* as dietary supplement (Jitsukawa, Suizu et al. 1984). In addition, protein components of *Chlorella* such as phenophorbide-a, a breakdown product of chlorophyll, and its ester recognized as photosensitizers may contribute to adverse reaction in the kidney (Yim, Yoo et al. 2007).. However, this photosensitization results

from ingestion of the algae in dietary supplements which is not relevant to exposures in this TERA field testing and is not a health concern for this assessment.

IV. HUMAN HEALTH HAZARDS OF THE SUBMISSION MICROORGANISMS

The concern for pathogenicity or toxicity associated with the introduced genes is low. As described by the submitters and the construct hazard analysis report, the introduced intergeneric DNA sequences for the sucrose non-fermenting (SNF) related kinase gene, *SNRK2*, is part of the serine/threonine kinases and plays a key role in sugar metabolism in plant and animal kingdoms and controls multiple growth and metabolic processes and is not expected to contribute pathogenicity or toxicity to the parental alga *C. sorokiniana*.

Another modification was the introduction of the *Streptoalloteichus hindustanus ble* gene that encodes resistance to the antibiotic zeocin. Zeocin is the commercial name for a special formulation of phleomycin D1 (Ceozin). It is a glycopeptide antibiotic of the bleomycin family (http://www.genaxxon.com/docs/pdf/zeocin_data.pdf). The *Sh ble* gene encodes resistance to zeocin, bleomycin, and phleomycin. Although these latter two antibiotics are inhibitory to a number of bacteria, they are only used clinically for their antitumor activity in reducing the growth of cancer cells. They are not used clinically in humans to treat infections. Thus, the usual concern regarding the loss of therapeutic value of an antibiotic if horizontal gene transfer of the antibiotic resistance gene to pathogens in the environment whose infections are treated with the antibiotic were to occur is not relevant to these antibiotics. Zeocin resistance is often used in microbial, plant, and animal transformations as a selection marker instead of clinically important antibiotics used to treat infections. Thus, there is low concern for use of the *ble* gene in the submission microorganisms.

V. RISK TO POTENTIALLY EXPOSED AND SUSCEPTIBLE SUBPOPULATIONS

Potential human health effects of the *C. sorokiniana* to susceptible subpopulations must be considered for pathogenicity, toxicity, and allergenicity. Susceptible subpopulations with respect to pathogenicity include those with not fully competent immune systems such as the young, the elderly, malnourished individuals, and those with pre-existing disease or on immunosuppressive therapy and populations that are highly exposed populations such as workers.

The three cases of chlorellosis reported were in compromised tissues of humans dermally exposed to contaminated waters. Thus, dermal exposure of open wounds to waters containing *Chlorella* may result in infection by *Chlorella.* However, in humans chlorellosis is very rare considering that only three cases have ever been reported and *Chlorella* is abundant world-wide including in fresh waters, marine waters, and in soil, so humans are routinely exposed to it.

In regards to toxicity, there is low concern for susceptible subpopulations as well as the general population as *Chlorella* is not known to produce phycotoxins.

There are low human health concerns to potentially exposed and susceptible subpopulations due to the genetic modifications made to these strains. Neither of these enzyme activities pose pathogenicity concerns to immunocompromised individual. Nor does the use of the zeocin resistance gene as zeocin is not used clinically to treat bacterial infections. The gene products of the introduced genes do not pose toxicity concerns to the general population or susceptible subpopulations.

VI. **CONCLUSIONS**

Overall, there is low human health concerns for the submission microorganism. No additional health concerns were identified for potentially exposed susceptible populations

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